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(54) DNA encoding a hyperthermstable alpha-amylase.

(57) [Object] To provide a genetic engineering process for producing  $\alpha$ -amylase with the use of an isolated hyperthermstable  $\alpha$ -amylase gene.

[Constitution] A hyperthermstable  $\alpha$ -amylase gene comprising a nucleotide sequence represented by SEQ ID NO: 1 of the sequence listing (obtained from *Pyrococcus furiosus* and having a sequence length of 1305). A hyperthermstable  $\alpha$ -amylase gene hybridizable to the above hyperthermstable  $\alpha$ -amylase gene. A process for producing a hyperthermstable  $\alpha$ -amylase by culturing a transformant into which a recombinant plasmid containing the above hyperthermstable  $\alpha$ -amylase gene has been introduced. At pH 5.5, the optimum temperature of the enzyme activity is in the range of 70 to 100 °C. The enzyme activity is entirely maintained even after heating at 80 °C for 6 hours, and at least 70% of the enzyme activity is maintained even after the heating at 95 °C for 6 hours.

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### [Field of Industrial Application]

This invention relates to a DNA encoding a hyperthermostable  $\alpha$ -amylase useful as an industrial enzyme.

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### [Prior Art]

The  $\alpha$ -amylase is being utilized in a variety of important industrial processes not only in the food industry but also in other various industries, such as the alcohol producing and textile industries. A typical use of the  $\alpha$ -amylase is found in a liquefaction of starch to be conducted in a glucose producing process for obtaining glucose, which is either processed into a sweetener and a nutriment or utilized as a starting material for isomerized sugar. The liquefied starch is also an important starting material for producing cyclodextrin and coupling sugar. Because of low solubility in water at medium temperature, starch is hardly hydrolyzed with  $\alpha$ -amylase. Consequently, liquefaction of starch with an  $\alpha$ -amylase is generally performed by first gelatinizing the starch at a temperature as high as about 100 °C and then contacting the resultant starch with the  $\alpha$ -amylase for liquefaction.

The pH value of the reaction mixture during the liquefaction is adjusted to an acidic region in order to suppress the formation of by-products. Especially, when the liquefaction is continuously followed by a saccharifying step, it is desired that the liquefaction be carried out in an acidic condition suitable for having glucoamylase employed in the saccharification fully exert its activities. Therefore, it is required that the  $\alpha$ -amylase for use in the liquefaction of starch have high activity even at extremely high temperatures and in acidic regions.

The liquefaction of starch is performed by the use of, for example,  $\alpha$ -amylase derived from Bacillus licheniformis (trade name: Termamyl, produced by Novo Nordisk) and  $\alpha$ -amylase derived from Bacillus subtilis (trade name: Speedase, produced by The Nagase & Co., Ltd.). Although these are mesophiles-derived enzymes, their thermostability is relatively high.

In this connection, it is anticipated that the hyperthermophilic bacteria adapted to high-temperature environment might have produced  $\alpha$ -amylases having increased thermostabilities and being suitable for use in the above liquefaction.

30 It is known that hyperthermophile *Pyrococcus furiosus* and *Pyrococcus woesei* produce  $\alpha$ -amylases [Applied and Environmental Microbiology, 56, 1985-1991 (1990); Archives of Microbiology, 155 (6), 572-578 (1991); FEMS Microbiology Letters, 71 (1-2), 21-26 (1990); and WO 90/11352].

### [Problem to be Solved by the Invention]

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The use of the above  $\alpha$ -amylases derived from bacteria of the genus Pyrococcus in the liquefaction of starch is anticipated because of their high thermostabilities. In the above documents, however, not only was the amount of the produced enzyme very small but also the culturing of the microorganism at high temperatures was inevitable for obtaining the enzyme. Therefore, the processes disclosed in these documents involved problems from the viewpoint of the use as industrial production processes. Further, in the above documents, the  $\alpha$ -amylases were not purified. Thus, they failed to provide purified enzymes.

In view of the above problems, the development of a genetic engineering process for producing the above  $\alpha$ -amylase is desired in the art.

The present invention are to isolate a DNA encoding  $\alpha$ -amylase derived from Pyrococcus furiosus and to provide a above-mentioned DNA, thereby solving the above problems.

### [Means for Solving the Problem]

50 In a aspect of the present invention, there is provided an isolated DNA selected from the group consisting of:

(a) a DNA encoding a hyperthermstable  $\alpha$ -amylase and having a nucleotide sequence selected from the group consisting of the sequences represented by SEQ ID No: 1, SEQ ID No: 2 and SEQ ID No: 3 of the sequence listing;

55 (b) a DNA capable of hybridizing to the DNA of (a) under stringent conditions and which encodes hyperthermophilic  $\alpha$ -amylase.

The stringent condition is as follows:

(1) A nylon membrane on which DNA is fixed is incubated with probe in the solution containing to final concentration 6xSSC (1xSSC contains 8.67g of NaCl and 4.41g of sodium citrate in one liter of water), 1%

SDS, 100 µg/ml of salmon sperm DNA, and 5xDenhardt's, which contains 0.1% bovine serum albumin, 0.1% polyvinyl-pirrolidone, and 0.1% Ficoll at 65 °C for 20 hours.

(2) The nylon membrane is first washed with 2xSSC containing 0.1% SDS at 37 °C and either washing temperature is increased every three degrees or SSC concentration is decreased every ten percents starting from the original condition until a signal derived from DNA on the membrane and that from background are distinguishable.

5 The inventors independently attempted to purify the  $\alpha$ -amylase derived from Pyrococcus furiosus DSM 3638 and determine the amino acid sequence of a part of the enzyme with a view toward obtaining the DNA encoding the  $\alpha$ -amylase. However, the purification of the enzyme was difficult, and the inventors failed to obtain an enzyme preparation having a purity that permit determination of the partial amino acid sequence.

10 The expression cloning method permits cloning of a gene of the enzyme in the absence of any information on the primary structure of the desired enzyme. For example, pullulanase gene was obtained from Pyrococcus woesei by this method (W0 92/02614). However, the method cannot be applied to cloning of any type of enzyme because in case the plasmid vector is used for the method, a very suitable 15 restriction enzyme is needed; It must cleave the target DNA into small size enough to be inserted in a plasmid vector and neither cleave the target gene at an inner site. Furthermore, the method is complicated because it needs a number of clones.

10 Subsequently, the present inventors have attempted to isolate the DNA encoding the  $\alpha$ -amylase by screening  $\alpha$ -amylase activity in a cosmid library constructed with Pyrococcus furiosus genomic DNA and a 20 cosmid vector, in which larger DNA fragments (35-50kb) can be inserted than in plasmid vectors. By using cosmid vectors, possibilities for cleaving an inner site of the target DNA encoding the enzyme by a restriction enzyme decrease and the numbers of clones necessary to test can be reduced. On the contrary, the cosmid vectors have less copy numbers in host organisms than the plasmid vectors, so that it may be difficult to detect the enzyme activity which is expressed at low level.

25 To inventors surprise, they realized that some cosmid clones did express proteins that are encoded by Pyrococcus furiosus DNA and also noticed that high thermostability of the target enzyme could be of great advantage for screening of its gene. Lysates of thermostable proteins was quite efficiently prepared being separated from thermolabile ones derived from host cells. The inventors cultivated each of the transformants in the cosmid library and prepared lysates containing only the thermostable proteins. The group of 30 these lysates is named as "cosmid protein library". By using the library thus constructed for detection of the enzyme activity, detection sensitivity increases than using colonies on a petri dish of the transformants and disadvantages such as background or inhibition of enzyme activity caused by proteins from hosts can be removed.

35 The present inventors isolated several cosmid clones which show  $\alpha$ -amylase activity by screening the cosmid protein library constructed with Pyrococcus furiosus genomic DNA. Furthermore, the present inventors isolated the gene coding a hyperthermostable  $\alpha$ -amylase from the DNA fragments inserted in the clones isolated above by making full use of various genetic engineering techniques, and determined the DNA sequence of the gene. And more, the present inventors succeeded in the expression of the hyperthermostable  $\alpha$ -amylase with the use of the gene, thus completing the present invention.

40 The expression cloning method using cosmid vectors described here cannot be always applied to any thermostable enzyme. The result is determined by the property of the target gene. For the example, the present inventors attempted to isolate the DNA encoding a protease of Pyrococcus furiosus (Applied and Environmental Microbiology, 56, 1992-1998 (1990)), but they didn't reach to the isolation of the gene.

45 It is known that most of the  $\alpha$ -amylases having undergone gene analysis are first synthesized as precursor proteins and, subsequently, converted to mature enzymes with the signal peptide cut off [Applied Microbiology and Biotechnology, 23, 355-360 (1986)].

50 The inventors have succeeded in producing the desired  $\alpha$ -amylase by determining the nucleotide sequence of an isolated DNA encoding hyperthermostable  $\alpha$ -amylase, comparing it with that of the known DNA encoding  $\alpha$ -amylase to thereby presume a region coding for the mature  $\alpha$ -amylase, constructing a recombinant plasmid capable of expressing the mature  $\alpha$ -amylase in a microorganism, and cultivating the microorganism transformed with the recombinant plasmid, and thus have completed the present invention.

55 The DNA encoding the hyperthermostable  $\alpha$ -amylase according to the present invention can be obtained by screening a gene library of hyperthermophiles. The hyperthermophile is, for example, a bacterium belonging to the genus Pyrococcus. Thus, for example, the desired gene can be obtained by screening a cosmid library of the genome of Pyrococcus furiosus. For this purpose, Pyrococcus furiosus DSM3638 can be employed, which is available from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH.

The cosmid library of the genome of Pyrococcus furiosus can be obtained by partially digesting the genomic DNA of Pyrococcus furiosus with a restriction enzyme Sau3A I (produced by Takara Shuzo Co., Ltd.), ligating the resultant DNA fragments to triple helix cosmid vector (produced by Stratagene), packaging in lambda phage particles by the in vitro packaging method, and transforming an appropriate strain of Escherichia coli, e.g., Escherichia coli DH5 $\alpha$ MCR (produced by BRL). A group of lysates containing thermostable proteins expressed in Escherichia coli, which is named cosmid protein library, can be prepared as follows: each of bacterial cells obtained by cultivating the transformants is heated at 100 °C for 10 minutes, sonicated, and heated at 100 °C for 10 minutes again, centrifuged, and supernatant is collected. The  $\alpha$ -amylase activity in this cosmid protein library is examined using starch as substrate. In this manner, some cosmid clones each containing a DNA encoding a hyperthermostable  $\alpha$ -amylase can be obtained which express  $\alpha$ -amylases resistant to the above heat treatment.

Further, a cosmid DNA prepared from the thus obtained cosmid clone containing the DNA encoding the hyperthermostable  $\alpha$ -amylase may be digested to some DNA fragments with appropriate restriction enzymes for construction of recombinant plasmids containing the above DNA fragments. The recombinant plasmids are introduced to an appropriate microorganism to thereby obtain transformants. The activity of the  $\alpha$ -amylase produced by each of the transformants is examined and the recombinant plasmid containing the desired DNA encoding the hyperthermostable  $\alpha$ -amylase can be obtained.

More specifically, the cosmid DNA prepared from one of the above cosmid clones is digested with Hind III (produced by Takara Shuzo Co., Ltd.), and the resultant DNA fragments are inserted in plasmid vector pUC119 (produced by Takara Shuzo Co., Ltd.) at its Hind III site. Subsequently, the recombinant plasmids are introduced to Escherichia coli JM109 (produced by Takara Shuzo Co., Ltd.), and the resultant transformants are grown on a nitrocellulose membrane. Cells on the membrane are lysed with chloroform vapor, and the membrane is put on an agarose gel containing starch and incubated. After the incubation, starch degradation is examined by the iodine-starch reaction to thereby detect the  $\alpha$ -amylase activity of each of the transformants. Thus, a recombinant plasmid containing a DNA encoding  $\alpha$ -amylase which express a hyperthermostable  $\alpha$ -amylase activity can be obtained.

The obtained plasmid was designated plasmid pH186, and the Escherichia coli JM109 transformed with the plasmid was designated as Escherichia coli JM109/H-86. This strain was deposited on July 16, 1993 at National Institute of Bioscience and Human-Technology Agency of Industrial Science and Technology (1-3, Higashi 1 chome Tsukuba-shi Ibaraki-ken 305, JAPAN), under the accession number FERM P-13759, and on July 29, 1994 this deposit was converted to deposit at National Institute of Bioscience and Human-Technology Agency of Industrial Science and Technology in accordance with the Budapest Treaty under the accession number FERM BP-4763. Fig. 1 shows the restriction map of the DNA fragment inserted in the plasmid pH186.

The plasmid pH186 is digested with various restriction enzymes to produce DNA fragments, and the DNA fragments are inserted in an appropriate plasmid vector to thereby obtain recombinant plasmids. Escherichia coli JM109 is transformed with the recombinant plasmids, and the  $\alpha$ -amylase activity of each of the transformants is examined. Thus, the DNA fragments which do not contain DNA encoding hyperthermostable  $\alpha$ -amylase can be deleted from the plasmid pH185. Specifically, the above plasmid pH186 is digested with Hind III and Sph I (produced by Takara Shuzo Co., Ltd.), and DNA fragment of about 2.4 kb is isolated and ligated to Hind III and Sph I sites of plasmid vector pTV118N (produced by Takara Shuzo Co., Ltd.). The obtained recombinant plasmid is introduced to Escherichia coli JM109. The  $\alpha$ -amylase activity of each of the resultant colonies is examined by the above method employed in the screening of the cosmid protein library, and the plasmid is prepared from the colony expressing the  $\alpha$ -amylase activity. The obtained recombinant plasmid was designated plasmid pSH24, and the Escherichia coli JM109 transformed with the plasmid was designated Escherichia coli JM109/pSH24. Fig. 2 shows the restriction map of the DNA fragment inserted in the plasmid pSH24.

The nucleotide sequence of the above plasmid pSH24 can be determined by any of the conventional methods, e.g., the dideoxy method.

Specifically, various deletion mutants of the plasmid pSH24 having part of the inserted DNA fragment are prepared, and the nucleotide sequence are determined by the dideoxy method using suitable ones among the deletion mutants as templates. Further, the nucleotide sequence of the DNA fragment containing the DNA encoding the hyperthermostable  $\alpha$ -amylase which has been inserted in the plasmid pSH24 can be determined by comparing and analyzing the each of the nucleotide sequences of the deletion mutants.

The nucleotide sequence of the Eae I-Hind III region of the DNA fragment inserted in the plasmid pSH24 is shown in SEQ ID NO: 3 of the sequence listing. That is, the SEQ ID NO : 3 of the sequence listing is an example of the nucleotide sequence of the DNA fragment containing the DNA encoding the hyperthermostable  $\alpha$ -amylase obtained according to the present invention. The amino acid sequence of the

hyperthermostable  $\alpha$ -amylase deduced from the above nucleotide sequence is shown in SEQ ID NO: 4 of the sequence listing.

A recombinant plasmid capable of expressing a hyperthermostable mature  $\alpha$ -amylase Escherichia coli can be constructed on the basis of the above amino acid sequence. That is, the position of the N-terminal 5 amino acid of the mature  $\alpha$ -amylase can be presumed by comparing the above amino acid sequence with various  $\alpha$ -amylases whose amino acid sequences are known [Applied Microbiology and Biotechnology, 23, 355-360 (1986)].

Oligonucleotide AMFN (shown in Fig. 3) having a nucleotide sequence shown in SEQ ID NO: 5 of the sequence listing, which can introduce the initiation codon (ATG) and the Nco I site (CCATGG) immediately 10 ahead of the codon for the above N-terminal amino acid of the mature  $\alpha$ -amylase on the plasmid pSH24, and oligonucleotide AMRS (shown in Fig. 4) having a nucleotide sequence shown in SEQ ID NO: 6 of the sequence listing, which is complementary to a region including the Spe I site of the plasmid pSH24, are synthesized. PCR is performed with the use of the above oligonucleotides as primers and the plasmid 15 pSH24 as a template, thereby amplifying a DNA fragment having the above two oligonucleotides at each terminals and including part of the DNA encoding the hyperthermostable  $\alpha$ -amylase. This DNA fragment is digested with Nco I (produced by Takara Shuzo Co., Ltd.) and Spe I (produced by Takara Shuzo Co., Ltd.), 20 ligated to about 4.6 kb Nco I-Spe I fragment of the plasmid pSH24, and introduced to Escherichia coli JM109. The resultant colonies are cultivated in the presence of IPTG, and the  $\alpha$ -amylase activity of each of the colonies is examined in accordance with the above method employed in the screening of cosmid 25 protein library. The plasmid is prepared from a colony expressing the  $\alpha$ -amylase activity.

The obtained plasmid was designated plasmid pNH17, and the Escherichia coli JM109 transformed with the plasmid was designated Escherichia coli JM109/pNH17. This strain was deposited on September 10, 1993 at National Institute of Bioscience and Human-Technology Agency of Industrial Science and Technology (1-3, Higashi 1 chome Tsukuba-shi Ibaraki-ken 305, JAPAN), under the accession number FERM P- 25 13859, and on July 29, 1994 This deposit was converted to deposit at National Institute of Bioscience and Human-Technology Agency of Industrial Science and Technology in accordance with the Budapest Treaty under the accession number FERM BP-4764.

Fig. 5 shows the restriction map of the DNA fragment inserted in the plasmid pNH17. In the figure, the bold arrow indicates the region coding for the hyperthermostable  $\alpha$ -amylase protein. The nucleotide 30 sequence of the DNA fragment inserted in the plasmid pNH17 is shown in SEQ ID NO: 2 of the sequence listing. That is, the SEQ ID NO: 2 of the sequence listing is an example of the nucleotide sequence of the DNA fragment containing the DNA encoding the hyperthermostable  $\alpha$ -amylase obtained according to the present invention.

Furthermore, the nucleotide sequence of the DNA fragment encoding the hyperthermostable  $\alpha$ -amylase, 35 which is illustrated with bold arrow in Fig. 5, is shown in SEQ ID NO: 1 of the sequence listing. That is, the SEQ ID NO: 1 of the sequence listing is the hyperthermostable  $\alpha$ -amylase gene obtained according to the present invention. The amino acid sequence of the mature hyperthermostable  $\alpha$ -amylase deduced from above nucleotide sequence is shown in SEQ ID NO: 7 of the sequence listing.

When Escherichia coli JM109/pNH17 is cultivated under conventional conditions, e.g., at 37 °C in LB 40 medium (1% tryptan, 0.5% yeast extract, and 0.5% NaCl; pH 7.0) containing 50  $\mu$ g/ml ampicillin and when IPTG is added at an appropriate time during the cultivation, the bacterial cells express the hyperthermostable  $\alpha$ -amylase. The bacterial cells are harvested from culture, sonicated, and centrifuged to thereby obtain a supernatant as a cell-free extract. The cell-free extract is heated at 95 °C for 30 minutes to denature the thermolabile proteins, and nucleic acid removal, salting-out with ammonium sulfate and 45 dialysis are carried out to thereby obtain a partially purified hyperthermostable  $\alpha$ -amylase preparation.

The enzymatic and physicochemical properties of the partially purified hyperthermostable  $\alpha$ -amylase produced by Escherichia coli JM109/pNH17 are as follows.

#### (1) Function

50 The  $\alpha$ -amylase is capable of hydrolyzing starch and thus reducing the coloration exhibited by the iodine-starch reaction. Further, it hydrolyzes and liquefies gelatinized starch.

#### (2) Assay for enzyme Activity

55 Enzyme activity was measured by spectrophotometrically following the reduction of coloration due to the iodine-starch reaction conducted after the enzymatic hydrolysis of soluble starch as a substrate. Specifically, the enzyme preparation to be assayed was appropriately diluted, and 50  $\mu$ l of the resultant

preparation was added to 200  $\mu$ l of 50 mM sodium phosphate buffer (pH 5.5) containing 0.5% soluble starch (produced by Merck & Co., Ltd.), and incubated at 95 °C for 15 minutes.

The reaction mixture was cooled on ice to thereby stop the reaction, and 500  $\mu$ l of a 0.01 N iodine solution (containing 1% potassium iodide) was added thereto and mixed. An aliquot of 50  $\mu$ l was withdrawn from the mixture, diluted 10-fold with distilled water, and the absorbance at 600 nm was measured.

One unit of the enzyme was defined as the amount of the enzyme with the absorbance at 600 nm was reduced at 95 °C by 1% per minute. The hyperthermstable  $\alpha$ -amylase preparation obtained according to the present invention exhibited a starch hydrolyzing activity when measured at 95 °C and at pH 5.5.

Further, the liquefaction activity of the enzyme was measured with the use of corn starch as a substrate. Specifically, 1.5 ml of 50 mM sodium phosphate buffer containing 50 units of the enzyme was added to 1.5 ml of 50 mM sodium phosphate buffer (pH 5.5) containing 20% corn starch (produced by Nacalai Tesque). The mixture was gelatinized with agitating on a boiling water bath, then incubated at 95 °C for 60 minutes. The viscosity of the reaction mixture markedly decreased as compared with that of a control in which the enzyme was not added. The hyperthermstable  $\alpha$ -amylase preparation obtained according to the present invention exhibited starch liquefaction activity when measured at 95 °C and at pH 5.5.

The enzymatic activity mentioned in the following items (3) to (5) was one determined by the above activity assay method utilizing the iodine-starch reaction.

#### (3) Optimum pH

In order to investigate the optimum pH, the enzyme activity was measured with substrate solution adjusted to various pH. The substrate solutions were prepared with the following buffers: 50 mM sodium acetate buffer for pH 3.5 to 6.0, 50 mM potassium phosphate buffer for pH 6.0 to 8.0, and 50 mM sodium borate buffer for pH 8.5 to 9.5. Fig. 6 shows the relationship between the pH and the activity of the hyperthermstable  $\alpha$ -amylase preparation obtained according to the present invention. The ordinate indicates the relative activity (%) of the enzyme while the abscissa indicates the pH of the reaction mixture in the figure. Further, in the figure, the open circle, the cross, and the open square, respectively, indicate the results obtained with the use of the 50 mM sodium acetate buffer, the 50 mM potassium phosphate buffer, and the 50 mM sodium borate buffer. It is apparent from Fig. 6 that the hyperthermstable  $\alpha$ -amylase preparation of the present invention is active at pH values ranging from 4.5 to 6.5 and exhibits the highest activity at pH 5.5.

#### (4) Optimum Temperature

The enzyme activities were measured at various temperatures to investigate the optimum temperature.

Fig. 7 shows the relationship between the temperature and the activity of the hyperthermstable  $\alpha$ -amylase preparation obtained according to the present invention. The ordinate indicates the relative activity (%) of the enzyme while the abscissa indicates the temperature (°C) of the reaction mixture in the figure. It is apparent from Fig. 7 that the hyperthermstable  $\alpha$ -amylase preparation of the present invention is highly active at temperatures ranging as broadly as 70 to 100 °C when measured at a pH value of 5.5.

#### (5) Thermostability

The enzyme solution was incubated at each of 80 and 95 °C for various periods, and the change of residual enzyme activity was measured to investigate the thermostability of the enzyme. Fig. 8 shows the thermostability of the hyperthermstable  $\alpha$ -amylase preparation obtained according to the present invention. The ordinate indicates the residual activity (%) of the enzyme while the abscissa indicates the time (hr) during which the enzyme solution was incubated in the figure. It is apparent from Fig. 8 that the activity of the hyperthermstable  $\alpha$ -amylase preparation of the present invention does not decrease even when the enzyme solution is incubated at 80 °C for 6 hours and at least 70% of the initial activity still remains even when the enzyme solution is incubated at 95 °C for 6 hours.

As described in detail above, the present invention provides the DNA encoding the hyperthermstable  $\alpha$ -amylase. Further, the present invention can produce the industrial process of the hyperthermstable  $\alpha$ -amylase with the use of the above DNA.

The  $\alpha$ -amylase encoded by the gene of the present invention can be used in a variety of important industrial processes not only in the food industry but also in other various industries, such as the alcohol producing and textile industries. A typical use of the  $\alpha$ -amylase encoded by the gene of the present invention is found in a liquefaction of starch to be conducted in a process for obtaining glucose, which can

be either processed into a sweetener and a nutriment or utilized as a starting material for an isomerized sugar. The liquefied starch is also an important starting material for producing cyclodextrin and coupling sugar. Liquefaction of starch with an  $\alpha$ -amylase encoded by the gene of the present invention can be performed by contacting starch directly with the  $\alpha$ -amylase immediately after gelatinizing the starch at a

5 temperature as high as about 100 °C.

The pH value of the reaction mixture during the liquefaction is adjusted to an acidic region in order to suppress the formation of by products. Especially, when the liquefaction is continuously followed by a saccharifying step, it is desired that the liquefaction be carried out in an acidic condition suitable for having glucoamylase employed in the saccharification fully exert its activities. Because the  $\alpha$ -amylase encoded by

10 the gene of the present invention have high activity even at extremely high temperatures and in acidic regions, it is quite useful for liquefaction of starch.

In addition, all of the gene which is similar, but not same to the gene obtained according to the present invention and which is expected to express the same enzyme activity may be obtained by hybridization in stringent condition using the gene, the DNA fragment containing the gene encoding the hyperthermstable

15  $\alpha$ -amylase obtained according to the present invention, or appropriate part of the nucleotide sequence of the gene as probe.

Moreover, all of the gene which is similar, but not same to the gene obtained according to the present invention and expected to express the same enzyme activity may be obtained by PCR using appropriate part of the nucleotide sequence of the gene encoding the hyperthermstable  $\alpha$ -amylase obtained according

20 to the present invention as primers.

Furthermore, the DNA encoding the hyperthermstable  $\alpha$ -amylase obtained according to the present invention may be appropriately modified so as to further improve the functions of the  $\alpha$ -amylase.

[Brief Description of the Drawings]

25

[Fig. 1]

A figure showing the restriction map of the DNA fragment of about 3.8 kb containing the DNA encoding

the hyperthermstable  $\alpha$ -amylase.

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[Fig. 2]

A figure showing the restriction map of the DNA fragment of about 2.4 kb containing the DNA encoding

the hyperthermstable  $\alpha$ -amylase.

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[Fig. 3]

A figure showing the sequence of oligonucleotide AMFN.

40 [Fig- 4]

A figure showing the sequence of oligonucleotide AMRS.

[Fig. 5]

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A figure showing the restriction map of the DNA fragment of about 1.7 kb containing the DNA encoding

the hyperthermstable  $\alpha$ -amylase.

[Fig. 6]

50

A figure showing the relationship between the pH and the activity of the hyperthermstable  $\alpha$ -amylase obtained according to the present invention.

[Fig. 7]

55

A figure showing the relationship between the temperature and the activity of the hyperthermstable  $\alpha$ -amylase obtained according to the present invention.

[Fig. 8]

A figure showing the thermostability of the hyperthermostable  $\alpha$ -amylase obtained according to the present invention.

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## [Examples]

Embodiments of the present invention will now be set out, which in no way limit the scope of the invention.

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In the Examples, % means % by weight.

## Example 1

(Preparation of Pyrococcus furiosus genome DNA)

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Pyrococcus furiosus DSM 3638 was incubated in the following manner.

Two l of a medium comprising 1% of tryptone, 0.5% of yeast extract, 1% of soluble starch, 3.5% of Jamarin S Solid (produced by Jamarin Laboratory), 0.5% of Jamarin S Liquid (produced by Jamarin Laboratory), 0.003% of MgSO<sub>4</sub>, 0.001% of NaCl, 0.0001% of FeSO<sub>4</sub> 7H<sub>2</sub>O, 0.0001% of CoSO<sub>4</sub>, 0.0001% of CaCl<sub>2</sub> 7H<sub>2</sub>O, 0.0001% of ZnSO<sub>4</sub>, 0.1 ppm of CuSO<sub>4</sub> 5H<sub>2</sub>O, 0.1 ppm of KAl(SO<sub>4</sub>)<sub>2</sub>, 0.1 ppm of H<sub>3</sub>BO<sub>3</sub>, 0.1 ppm of Na<sub>2</sub>MoO<sub>4</sub> 2H<sub>2</sub>O, and 0.25 ppm of NiCl<sub>2</sub> • 6H<sub>2</sub>O was fed into a 2 l medium bottle and sterilized at 120 °C for 20 minutes. After eliminating the dissolved oxygen by blowing nitrogen gas, the medium was inoculated with the above-mentioned strain, which was then stationarily incubated at 95 °C for 16 hours. After the completion of the incubation, cells were collected by centrifugation.

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Then the collected cells were suspended in 4 ml of a 0.05 M Tris-HCl (pH 8.0) containing 25% of sucrose. To the obtained suspension were added 0.8 ml of lysozyme [5 mg/ml, 0.25 M Tris-HCl (pH 8.0)] and 2 ml of 0.2 M EDTA. After maintaining at 20 °C for 1 hour, 24 ml of an SET solution [150 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl (pH 8.0)] was added. Further, 4 ml of 5% SDS and 400  $\mu$ l of proteinase K (10 mg/ml) were added thereto, followed by a reaction at 37 °C for 1 hour. After the completion of the reaction, the reaction mixture was extracted with chloroform/phenol and precipitated with ethanol. Thus approximately 3.2 mg of a genome DNA was prepared.

## (Preparation of cosmid protein library)

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400  $\mu$ g of the Pyrococcus furiosus DSM 3638 genomic DNA was partially digested with Sau3A I and fractionated according to the size by density gradient centrifugation. One  $\mu$ g of Triple Helix Cosmid Vector was cleaved with BamH I and mixed with 140  $\mu$ g of the genomic DNA fragments of 35 to 50 kb which had been obtained by the fractionation as described above. After ligating with the use of a Ligation Kit (produced by Takara Shuzo Co., Ltd.), the Pyrococcus furiosus genomic DNA fragments were packaged into lambda phage particles by the in vitro packaging method using Gigapack II Gold (produced by Stratagene). By using a part of the phage solution thus obtained, Escherichia coli DH5 $\alpha$ MCR was transformed to thereby give a cosmid library.

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First cosmid DNA was prepared from several colonies thus obtained and it was confirmed that they had an inserted fragment of an appropriate size in common. Next, each of 500 colonies was suspended in 150 ml of LB medium containing 100  $\mu$ g/ml of ampicillin and incubated under shaking at 37 °C for 16 hours. The culture was centrifuged and cells were collected as precipitate. These cells were suspended in 20 mM Tris-HCl (pH 8.0) and treated at 100 °C for 10 minutes. Subsequently, they were sonicated and further treated at 100 °C for 10 minutes.

The lysate obtained as supernatant after centrifugation was used as a cosmid protein library.

50

(Selection of cosmid clones containing DNA encoding hyperthermostable  $\alpha$ -amylase)

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The  $\alpha$ -amylase activity was detected by spectro-photometrically following the reduction of coloration due to the iodine-starch reaction which occurred by the hydrolysis of starch. Specifically, 20  $\mu$ l of lysate were withdrawn from the above cosmid protein library, and 50  $\mu$ l of 0.25 M sodium phosphate buffer containing 0.04% of soluble starch (pH 5.5) was added thereto. The mixture was incubated at 95 °C for 30 minutes, and 50  $\mu$ l of 0.01 N iodine solution (containing 1% potassium iodide) and 150  $\mu$ l of distilled water were added thereto. Thereafter, the absorbance at 630 nm was measured and compared with that of a

control. Thus, nine cosmid clones having  $\alpha$ -amylase activities were obtained from the cosmid protein library.

(Construction of plasmid pH186)

5 A cosmid DNA was prepared from one of the nine cosmid clones which exhibited the highest  $\alpha$ -amylase activity, digested with each of BamH I, EcoR I, Hind III, and Pst I, and ligated to the multicloning site of a plasmid vector pUC119. The resultant recombinant plasmid was introduced to Escherichia coli JM109 and spread on an LB plate containing 50  $\mu$ g/ml ampicillin. The  $\alpha$ -amylase activity of each of the thus formed colonies was measured. Namely, the formed colonies were transferred onto a nitrocellulose membrane, and incubated on an LB plate containing 50  $\mu$ g/ml ampicillin at 37°C overnight. Subsequently, the membrane was incubated in chloroform vapor for 15 minutes to thereby cause the colonies to undergo bacteriolysis. The resultant membrane was dried in air and placed, with its colony side down, on an agarose plate containing 1% soluble starch and a 50 mM sodium phosphate buffer (pH 5.5). After the incubation at 70°C overnight, the membrane was removed and a 0.01 N iodine solution (containing 1% potassium iodide) 10 was applied to the plate to thereby color the starch. Colonies having  $\alpha$ -amylase activities, which had no coloration with the iodine solution occurred because of the hydrolysis of starch were selected.

15

Colonies exhibiting the  $\alpha$ -amylase activity were found among the colonies of the transformants prepared with the use of the three restriction enzymes exclusive of BamH I. Plasmid was prepared from each of the colonies. The size of the DNA fragment inserted in each of the resultant plasmids was determined. The 20 recombinant plasmids constructed with EcoR I, Hind III, and Pst I contained DNA fragments of about 6.5, about 3.8, and about 8 kb, respectively. The recombinant plasmid containing the smallest DNA fragment which was constructed with Hind III was designated pH186. The Escherichia coli JM109 transformed with the above plasmid was designated Escherichia coli JM109/H-86. This strain was deposited on July 16, 1993 at National Institute of Bioscience and Human-Technology Agency of Industrial Science and Technology (1-3, 25 Higashi 1 chome Tsukuba-shi Ibaraki-ken 305, JAPAN), under the accession number FERM P-13759, and on July 29, 1994. This deposit was converted to deposit at National Institute of Bioscience and Human-Technology Agency of Industrial Science and Technology in accordance with the Budapest Treaty under the accession number FERM BP-4763. Fig. 1 shows the restriction map of the DNA fragment inserted in the plasmid pH186.

30

(Construction of plasmid pSH24)

The above plasmid pH186 was digested with Hind III and Sph I, and a DNA fragment of about 2.4 kb 35 was isolated with agarose gel electrophoresis. This DNA fragment was ligated to a plasmid vector pTV118N digested with Hind III and Sph I, and introduced to Escherichia coli JM109. The  $\alpha$ -amylase activity of each of the formed colonies was examined by the method employed in the screening of the cosmid protein library.

A plasmid was prepared from the colony having exhibited  $\alpha$ -amylase activities, and designated plasmid pSH24. The Escherichia coli JM109 transformed with the above plasmid pSH24 was designated Escherichia coli JM109/pSH24. Fig. 2 shows the restriction map of the DNA fragment inserted in the plasmid pSH24.

Example 2

(Determination of nucleotide sequence of DNA encoding the hyperthermstable  $\alpha$ -amylase)

45

A series of deletion mutants were constructed from the plasmid pSH24 in order to determine the nucleotide sequence of the DNA fragment containing the DNA encoding the hyperthermstable  $\alpha$ -amylase. A Kilo sequence deletion kit (produced by Takara Shuzo Co., Ltd.) was used in the construction of the 50 deletion mutants. Some containing inserted DNA fragments with appropriate lengths were selected from among the deletion mutants, and the nucleotide sequence of each of the insert moieties was analyzed by the dideoxy method using the BcaBEST dideoxy sequencing kit (produced by Takara Shuzo Co., Ltd.). The data were combined and, consequently, the nucleotide sequence of the DNA fragment inserted in the plasmid pSH24 was determined. SEQ ID NO: 3 of the sequence listing shows nucleotide sequence from Eae I site to Hind III site with 2134 bp long as part of the nucleotide sequence of the DNA fragment 55 containing the DNA encoding the hyperthermstable  $\alpha$ -amylase, which was inserted in the plasmid pSH24.

Similarity exists between the nucleotide sequence from position 337 to 373 of the above sequence and the promoter sequence of archaebacteria [Nucleic Acids Research, 14, 2459-2479 (1986); and Nucleic Acids Research, 16, 1-19 (1988)], and SD-like sequence exists between position 380 and 388 of the above

nucleotide sequence. Therefore, it is suggested that the hyperthermostable  $\alpha$ -amylase is coded for with the GTG starting with the G at position 395 of the above nucleotide sequence as an initiation codon. On the other hand, the termination codon is TGA starting with the T at position 1775 of the above nucleotide sequence. The amino acid sequence of the hyperthermostable  $\alpha$ -amylase deduced from the above 5 nucleotide sequence is shown in SEQ ID NO: 4 of the sequence listing.

All of four highly homologous regions found in the known  $\alpha$ -amylases [Applied Microbiology and Biotechnology, 23, 355-360 (1986)] were present in the above amino acid sequence. It is apparent from comparison of the N terminal region that 25 amino acid sequence, from Met 1 to Ala 25, of the above amino acid sequence correspond to a signal peptide, and that the N terminal residue of the mature  $\alpha$ -amylase is the  
10 Ala 26.

#### (Preparation of plasmid pNH17)

15 A recombinant plasmid capable of expressing a hyperthermstable mature  $\alpha$ -amylase in Escherichia coli was constructed on the basis of the above obtained results by the site-directed mutagenesis utilizing PCR. First, oligonucleotide AMFN (SEQ ID NO: 5) so designed as to introduce the Nco I site and initiation codon immediately ahead of the codon for the N terminal amino acid of the mature  $\alpha$ -amylase as shown in Fig. 3, and oligonucleotide AMRS (SEQ ID NO: 6) complementary to the region containing the Spe I site of the hyperthermstable  $\alpha$ -amylase gene as shown in Fig. 4, were chemically synthesized. A DNA fragment of about 300 bp having the above oligonucleotide sequences at each ends was amplified by PCR in which the above two oligonucleotides were used as primers and the plasmid pSH24 was used as template. Thus amplified DNA fragment was digested with Nco I and Spe I. Separately, the plasmid pSH24 was digested with Nco I and Spe I, and a DNA fragment of about 4.6 kb was isolated therefrom by agarose gel electrophoresis. This DNA fragment was ligated to the above PCR product digested with restriction enzymes and introduced to Escherichia coli JM109.

The resultant colonies were cultivated in the presence of IPTG and the  $\alpha$ -amylase activities thereof were examined by the method employed in the screening of the cosmid protein library. A plasmid was prepared from the colonies having exhibited activities and designated plasmid pNH17. The Escherichia coli JM109 transformed with the plasmid pNH17 was designated as Escherichia coli JM109/pNH17. This strain was deposited on September 10, 1993 at National Institute of Bioscience and Human-Technology Agency of Industrial Science and Technology (1-3, Higashi 1 chome Tsukuba-shi Ibaraki-ken 305, JAPAN), under the accession number FERM P-13859, and on July 29, 1994 this deposit was converted to deposit at National Institute of Bioscience and Human-Technology Agency of Industrial Science and Technology in accordance with the Budapest Treaty under the accession number FERM BP-4764. Fig. 5 shows the restriction map of the DNA fragment inserted in the plasmid pNH17. The nucleotide sequence of the DNA fragment inserted in the plasmid pNH17 is shown in SEQ ID NO: 2 of the sequence listing.

Furthermore, the coding region for the hyperthermophilic  $\alpha$ -amylase is illustrated with bold arrow in Fig. 5 and the nucleotide sequence of this part is shown in SEQ ID NO: 1 of the sequence listing. The amino acid sequence of the mature hyperthermophilic  $\alpha$ -amylase deduced from above sequence is shown in SEQ ID NO: 7 of the sequence listing.

### Example 3

### (Preparation of Partially purified hyperthermostable $\alpha$ -amylase)

45 The Escherichia coli JM109/pNH17 obtained in Example 2 was cultivated aerobically in 5 ml of an LB  
medium (1% trypton, 0.5% yeast extract, and 0.5% NaCl; pH 7.0) containing 50 µg/ml ampicillin at 37 °C  
overnight. 500 ml of the above medium was prepared in a 2 l Erlenmeyer flask, and inoculated with 5 ml of  
50 the above culture. The mixture was cultivated aerobically at 37 °C and when the absorbance at 660 nm of  
the culture reached 0.5, IPTG was added to a final concentration of 2 mM. The mixture was further  
cultivated at 37 °C for 12 hours. Cells were harvested from culture by centrifugation and suspended in 5 ml  
of a 50 mM sodium phosphate buffer (pH 5.5). The suspension was sonicated to disrupt the cells and  
centrifuged to obtain a cell-free extract. The cell-free extract was heated at 95 °C for 30 minutes and  
centrifuged to remove denatured protein. Streptomycin sulfate was added to the resultant supernatant to a  
55 final concentration of 1%.

The thus obtained solution was re-centrifuged and ammonium sulfate was added to the resultant supernatant to 80% saturation. The formed precipitates were recovered by centrifugation, dissolved in 1 ml of a 50 mM sodium phosphate buffer (pH 5.5), and dialyzed against the above buffer overnight. Thus,

partially purified enzyme preparation was obtained as the dialysate.

[Effect of the Invention]

5 The gene coding for  $\alpha$ -amylase having surprisingly high thermostability was obtained by virtue of the present invention. The hyperthermostable  $\alpha$ -amylase of high purity can be supplied in large quantities by the use of the above gene.

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## Sequence Listing

5           SEQ ID NO:1

SEQUENCE LENGTH: 1305

SEQUENCE TYPE:nucleic acid

10           STRANDEDNESS:double

TOPOLOGY:linear

15           MOLECULE TYPE:Genomic DNA

19           HYPOTHETICAL:NO

ANTI-SENSE:NO

20           ORIGINAL SOURCE:

ORGANISM:Pyrococcus furiosus

SEQUENCE DESCRIPTION:SEQ ID NO:1:

25           GCAAAATACT TGGAGCTTGA AGAGGGAGGA GTTATAATGC AAGCATTCTA TTGGGATGTT   60  
 CCAGGGGGAG GAATTGGTG GGATCATATA AGATCGAAGA TTCCCTGAATG GTATGAAGCT   120  
 GGAATCTCTG CAATATGGCT ACCTCCACCA ACCAAGGGGA TGAGTGGAGG ATATTCAATG   180  
 30           GGCTACGATC CCTATGATTA CTTGATCTC GCGAGTACT ACCAGAAGGG AACTGTAGAG   240  
 ACGCGTTTTG GATCAAAAGA AGAACTAGTG AGATTGATAC AAAC TGCCCCTATGGA   300  
 ATAAAGGTAA TCGCCGATGT AGTTATAAAC CACAGGGCTG GTGGTACCT AGAATGGAAC   360  
 35           CCCTTCGTTG GAGATTACAC ATGGACAGAC TTTTCTAAAG TTGCCTCAGG GAAATATACA   420  
 GCTAACTATC TGGACTTCCA TCCAAACGAG CTTCATGTT GTGACGAAGG AACCTTTGGA   480  
 GGATTTCCAG ATATATGTCA TCACAAAGAG TGGGATCAGT ACTGGCTATG GAAGAGCAAT   540  
 40           GAGAGTTATG CTGCTTATTT AAGAAGCATA GGATTTGATG GTTGGAGATT TGACTATGTT   600  
 AAGGGCTATG GAGCTTGGGT TGTAGAGAC TGGCTTAATT GGTGGGGAGG TTGGGCAGTT   660  
 GGAGAGTACT GGGACACAAA TGTAGATGCA CTACTAAGCT GGGCATATGA GAGTGGTGCA   720  
 45           AAGGTCTTTG ACTTCCCGCT CTACTATAAA ATGGATGAAG CATTGACAA TAACAACATT   780  
 CCAGCATTAG TCTATGCCCT ACAAAACGGA CAAACTGTAG TTTCCAGAGA TCCATTAAAG   840  
 GCAGTAACCT TCGTTGCCAA TCATGACACA GATATAATAT GGAACAAGTA TCCAGCATAT   900  
 50           GCGTTCATAT TGACATATGA GGGACAGCCA GAAATATTCT ACAGGGACTT TGAGGAATGG   960

CTGAACAAGG ATAAGCTAAT TAACCTCATT TGGATCCATG ATCATTGGC AGGAGGAAGC 1020  
 5 ACAACAATTG TCTACTACGA CAACGATGAG CTCATATTG TGAGAAATGG AGATTCTAGA 1080  
 AGGCCTGGC TTATAACTTA CATTAACTTG AGCCCTAACT GGCTTGGTAG GTGGGTATAC 1140  
 GTTCCAAAGT TTGCAGGGGC TTGTATTAT GAATACACTG GAAACCTAGG AGGATGGGTA 1200  
 10 GATAAAAGAG TAGATACTAG CGGATGGGTA TACCTAGAGG CACCACCTCA CGATCCAGCT 1260  
 AACGGCTACT ATGGGTACTC CGTATGGAGT TATTGTGGTG TTGGG 1305

SEQ ID NO:2

15 SEQUENCE LENGTH: 1670  
 20 SEQUENCE TYPE:nucleic acid  
 STRANDEDNESS:double  
 25 TOPOLOGY:linear  
 MOLECULE TYPE:Genomic DNA  
 HYPOTHETICAL:NO  
 30 ANTI-SENSE:NO  
 ORIGINAL SOURCE:

ORGANISM:Pyrococcus furiosus  
 35 SEQUENCE DESCRIPTION:SEQ ID NO:2:  
 CCATGGCAAA ATACTTGGAG CTTGAAGAGG GAGGAGTTAT AATGCAAGCA TTCTATTGGG 60  
 ATGTTCCAGG GGGAGGAATT TGGTGGGATC ATATAAGATC GAAGATTCT GAATGGTATG 120  
 AAGCTGGAAT CTCTGCAATA TGGCTACCTC CACCAAGCAA GGGGATGAGT GGAGGATATT 180  
 40 CAATGGGCTA CGATCCCTAT GATTACTTTG ATCTGGCGA GTACTACCAG AAGGAACTG 240  
 TAGAGACCGG TTTTGGATCA AAAGAAGAAC TAGTGAGATT GATACAAACT GCCCATGCCT 300  
 ATGGAATAAA GGTAATCGCC GATGTAGTTA TAAACCACAG GGCTGGTGGT GACCTAGAAT 360  
 GGAACCCCTT CGTTGGAGAT TACACATGGA CAGACTTTTC TAAAGTTGCC TCAGGGAAAT 420  
 45 ATACAGCTAA CTATCTGGAC TTCCATCCAA ACGAGCTCA TTGTTGTGAC GAAGGAACT 480  
 TTGGAGGATT TCCAGATATA TGTCACTACA AAGAGTGGGA TCAGTACTGG CTATGGAAGA 540  
 GCAATGAGAG TTATGCTGCT TATTTAAGAA GCATAGGATT TGATGGTTGG AGATTTGACT 600  
 50 ATGTTAAGGG CTATGGAGCT TGGGTTGTCA GAGACTGGCT TAATTGGTGG GGAGGTTGGG 660

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5 CAGTTGGAGA GTACTGGGAC ACAATGTA ATGCACTACT AACCTGGCA TATGAGACTG 720  
GTGCAAAGGT CTTGACTTC CCGCTCTACT ATAAAATGGA TGAAGCATT GACAATAACA 780  
ACATTCCAGC ATTACTCTAT GCCCTACAAA ACGGACAAAC TGTAGTTCG AGAGATCCAT 840  
TTAAGGCAGT AACTTTCGTT GCCAATCATG ACACAGATAT AATATGGAAC AAGTATCCAG 900  
CATATGGCTT CATATTGACA TATGAGGGAC AGCCAGTAAT ATTCTACAGG GACTTTGAGG 960  
10 AATGGCTGAA CAAGGATAAG CTAATTAACC TCATTTGGAT CCATGATCAT TTGGCAGGAG 1020  
GAAGCACAAC AATTGTCTAC TACGACAACG ATGAGCTCAT ATTGTGAGA AATGGAGATT 1080  
CTAGAAGGCC TGGGCTTATA ACTTACATTA ACTTGAGCCC TAACTGGGTT GGTAGGTGGG 1140  
15 TATACTGTTCC AAAGTTGCA GGGGCTTGTA TTCATGAATA CACTGGAAAC CTAGGAGGAT 1200  
GGGTAGATAA AAGAGTAGAT AGTACCGGAT GGGTATACCT AGAGGCACCA CCTCACGATC 1260  
CAGCTAACGG CTACTATGGG TACTCCGTAT GGAGTTATTG TGGTGTGGG TGACTTTTC 1320  
TTTTTCTTT TTAACAATGG GAGAACTGCA AATACTGCGA CAATTCCCTGG GCGGCATACA 1380  
20 GGAGTTCTG GAGCGGCTTC ATTCAATATT ACTGTTTTAT TTCCAATTCC ATACAACGTG 1440  
ACATTGTTG GTTGGAGAGG TTCCCAGGGA AGTCCCTTAT ATAGTTGAAC TGTAAAAGTT 1500  
TTGTTTCTGG GGAGGGGAAC CACGGCAAAG TACTGTCCCG TTGGAGATT CAAGAGGGT 1560  
25 CTGGCTCTG AGAGTTCTGG TATGTTCCCC TTGACCCAAA GGTAAACCACT GTACTTTCT 1620  
TGTAGGACTA TTAGTGCTGG GATATCATTG CTAGAATAGT ACCTAACGTT 1670

SEQ ID NO:3

35 SEQUENCE LENGTH: 2134

SEQUENCE TYPE:nucleic acid

## STRANDEDNESS:double

40 TOPOLOGY: Linear

MOLECULE TYPE: Genomic DNA

**HYPOTHETICAL: NO**

45 ANTI-SENSE: NO

ORIGINAL SOURCE:

50 ORGANISM: Pyrococcus furiosus  
SEQUENCE DESCRIPTION: SEQ ID NO: 3

CGGCCACCAT TGTAAACGTCA TCGTGAGTTG GCCCCGAGGCC TCCCCGAAATA ATAAGAACAT 60  
 CGGGTTTTCT GTTAAGGGAT TCTAAAATTA CAGACCTTAT ATCCTCCACG TCATCGCCAA 120  
 5 CAGTAGTTAT TCTCTTAACG AGGTATCCTC TCTCCGTTAA TTTCTTCGCT ATGTGTGCTG 180  
 AGTTGCTGTT TACAGTATTG CCTGTTAAA GTTCATCACC TACGGTAATT ATTTCCGCGA 240  
 10 ACATTGGTTC TCCCCAGGAA TTGTTTTAT CAAGAGTTA TTAGATTTG ACGTGCGTTG 300  
 ATGAACATTT ATGTTCACAT GATCATAACA GAAAAATTTA TATGTATCAT CACCAAGTGAT 360  
 ACATTATGAG ACTTTGGTGT ATGGAGGTGA TCACGTGAAC ATAAAGAAAT TAACACCCCT 420  
 15 CCTAACTCTA TTACTGTTT TTATAGTACT ACCAAGTCCA GTAAGTGCAG CAAAATACTT 480  
 CGAGCTTGAA GAGGGAGGAG TTATAATGCA AGCATTCTAT TGGGATGTTG CAGGGGGAGG 540  
 AATTTGGTGG GATCATATAA GATCGAAGAT TCCTGAATGG TATGAAGCTG GAATCTCTGC 600  
 20 AATATGGCTA CCTCCACCAA GCAAGGGGAT GAGTGGAGGA TATTCAATGG GCTACGATCC 660  
 CTATGATTAC TTTGATCTCG GCGAGTACTA CCAGAAGGGA ACTGTAGAGA CGCGTTTTGG 720  
 ATCAAAAGAA GAACTACTGTA GATTGATACA AACTGCCAT GCCTATGGAA TAAAGGTAAT 780  
 25 CGCCGATGTA GTTATAAACCC ACAGGGCTGG TGGTGACCTA GAATGGAACC CCTTCGTTGG 840  
 AGATTACACA TGGACAGACT TTTCTAAAGT TGCCTCAGGG AAATATACAG CTAACTATCT 900  
 GGACTTCCAT CCAAACGAGC TTCATTGTTG TGACGAAGGA ACCTTGGAG GATTTCAGA 960  
 30 TATATGTCAT CACAAAGAGT GGGATCAGTA CTGGCTATGG AAGAGCAATG AGAGTTATGC 1020  
 TGCTTATTTA AGAACCATAG GATTTGATGG TTGGAGATT GACTATGTTA AGGGCTATGG 1080  
 AGCTTGGTT GTCAGAGACT GGCTTAATTG GTGGGGAGGT TGGCAGTTG GAGAGTACTG 1140  
 35 GGACACAAAT GTAGATGCCAC TACTAAGCTG GGCATATGAG ACTGGTGCAA AGGTCTTTGA 1200  
 CTTCCCGCTC TACTATAAAA TGGATGAAGC ATTTGACAAT AACAAACATTC CAGCATTAGT 1260  
 CTATGCCCTA CAAAACGGAC AACTGTAGT TTGGAGAGAT CCATTTAAGG CAGTAACCTT 1320  
 40 CGTTGCCAAT CATGACACAG ATATAATATG GAACAAGTAT CCAGCATATG CGTTCATATT 1380  
 GACATATGAG GGACAGCCAG TAATATTCTA CAGGGACTT GAGGAATGCC TGAACAAGGA 1440  
 TAAGCTAATT AACCTCATTG GGATCCATGA TCATTTGCCA GGAGGAAGCA CAACAATTGT 1500  
 45 CTACTACGAC AACGATGAGC TCATAATTGT GAGAAATGGA GATTCTAGAA GGCCTGGGCT 1560  
 TATAACTTAC ATTAACCTGAA GCCCTAACTG GGTTGGTAGG TGGGTATACG TTCCAAAGTT 1620  
 TGCAGGGGCT TGTATTCTG AATACACTGG AAACCTAGGA GGATGGGTAG ATAAAAGAGT 1680  
 50 AGATAGTAGC GGATGGGTAT ACCTAGAGGC ACCACCTCAC GATCCAGCTA ACGGCTACTA 1740

TGGGTACTCC GTATGGAGTT ATTGTGGTGT TGGGTGACTT TTTCTTTTT CTTTTAACCA 1800  
 ATGGGAGAAAG TGCAAATACT GCGACAATTG CTGGGCCGCA TACAGGAGTT TCTGGAGCGG 1860  
 5 CTTCATTCAA TATTACTGTT TTATTTCAA TTCCATACAA CGTGACATTG GTTGGTTGGA 1920  
 GAGGTTCCCA GGGAACTCCC TTATATAGTT GAACTGTAAA AGTTTGTTT CTGGGGAGGG 1980  
 10 GAACCACGGC AACTAGTGT CCCGTTGGAG ATTTCAAGAG GGGTCTCGGC TCTGAGAGTT 2040  
 CTGGTATGTT CCCCTTGACC CAAAGGTAAC CAGTGTAGTT TCCTTGTAGG ACTATTAGT 2100  
 CTGGGATATC ATTGCTAGAA TAGTACCTAA GCTT 2134

15 SEQ ID NO:4

SEQUENCE LENGTH: 460

20 SEQUENCE TYPE: amino acid

STRANDEDNESS: single

TOPOLOGY: linear

25 MOLECULE TYPE: peptide

ORIGINAL SOURCE:

ORGANISM: Pyrococcus furiosus

30 SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met	Asn	Ile	Lys	Lys	Leu	Thr	Pro	Leu	Leu	Thr	Leu	Leu	Phe	
1													15	
Phe	Ile	Val	Leu	Ala	Ser	Pro	Val	Ser	Ala	Ala	Lys	Tyr	Leu	Glu
													30	
35														
Leu	Glu	Glu	Gly	Gly	Vai	Ile	Met	Gln	Ala	Phe	Tyr	Trp	Asp	Val
40														
Pro	Gly	Gly	Gly	Ile	Trp	Trp	Asp	His	Ile	Arg	Ser	Lys	Ile	Pro
45														
Glu	Trp	Tyr	Glu	Ala	Gly	Ile	Ser	Ala	Ile	Trp	Leu	Pro	Pro	Pro
50														
Ser	Lys	Gly	Met	Ser	Gly	Gly	Tyr	Ser	Met	Gly	Tyr	Asp	Pro	Tyr
55														
80														
85														
90														

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Asp Tyr Phe Asp Leu Gly Glu Tyr Tyr Gln Lys Gly Thr Val Glu  
 5 95 100 105  
 Thr Arg Phe Gly Ser Lys Glu Glu Leu Val Arg Leu Ile Gln Thr  
 110 115 120  
 Ala His Ala Tyr Gly Ile Lys Val Ile Ala Asp Val Val Ile Asn  
 10 125 130 135  
 His Arg Ala Gly Gly Asp Leu Glu Trp Asn Pro Phe Val Gly Asp  
 15 140 145 150  
 Tyr Thr Trp Thr Asp Phe Ser Lys Val Ala Ser Gly Lys Tyr Thr  
 155 160 165  
 Ala Asn Tyr Leu Asp Phe His Pro Asn Glu Leu His Cys Cys Asp  
 20 170 175 180  
 Glu Gly Thr Phe Gly Gly Phe Pro Asp Ile Cys His His Lys Glu  
 25 185 190 195  
 Trp Asp Gln Tyr Trp Leu Trp Lys Ser Asn Glu Ser Tyr Ala Ala  
 200 205 210  
 Tyr Leu Arg Ser Ile Gly Phe Asp Gly Trp Arg Phe Asp Tyr Val  
 30 215 220 225  
 Lys Gly Tyr Gly Ala Trp Val Val Arg Asp Trp Leu Asn Trp Trp  
 35 230 235 240  
 Gly Gly Trp Ala Val Gly Glu Tyr Trp Asp Thr Asn Val Asp Ala  
 245 250 255  
 40 Leu Leu Ser Trp Ala Tyr Glu Ser Gly Ala Lys Val Phe Asp Phe  
 260 265 270  
 Pro Leu Tyr Tyr Lys Met Asp Glu Ala Phe Asp Asn Asn Asn Ile  
 45 275 280 285  
 Pro Ala Leu Val Tyr Ala Leu Gln Asn Gly Gln Thr Val Val Ser  
 50 290 295 300  
 Arg Asp Pro Phe Lys Ala Val Thr Phe Val Ala Asn His Asp Thr

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	305	310	315
5	Asp Ile Ile Trp Asn Lys Tyr Pro Ala Tyr Ala Phe Ile Leu Thr		
	320	325	330
	Tyr Glu Gly Gln Pro Val Ile Phe Tyr Arg Asp Phe Glu Glu Trp		
	335	340	345
10	Leu Asn Lys Asp Lys Leu Ile Asn Leu Ile Trp Ile His Asp His		
	350	355	360
	Leu Ala Gly Gly Ser Thr Thr Ile Val Tyr Tyr Asp Asn Asp Glu		
15	365	370	375
	Leu Ile Phe Val Arg Asn Gly Asp Ser Arg Arg Pro Gly Leu Ile		
	380	385	390
20	Thr Tyr Ile Asn Leu Ser Pro Asn Trp Val Gly Arg Trp Val Tyr		
	395	400	405
	Val Pro Lys Phe Ala Gly Ala Cys Ile His Glu Tyr Thr Gly Asn		
25	410	415	420
	Leu Gly Gly Trp Val Asp Lys Arg Val Asp Ser Ser Gly Trp Val		
	425	430	435
30	Tyr Leu Glu Ala Pro Pro His Asp Pro Ala Asn Gly Tyr Tyr Gly		
	440	445	450
	Tyr Ser Val Trp Ser Tyr Cys Gly Val Gly		
35	455	460	

SEQ ID NO:5

SEQUENCE LENGTH: 25

SEQUENCE TYPE:nucleic acid

## 15 STRANDEDNESS:single

## TOPOLOGY: linear

MOLECULE TYPE: Other nucleic acid (synthetic DNA)

SEQUENCE DESCRIPTION: SEQ\_ID NO:5:

CACTAAGTGC CATGGCAAAA TACTT 25

5 SEQ ID NO:6  
SEQUENCE LENGTH: 25  
SEQUENCE TYPE:nucleic acid  
10 STRANDEDNESS:single  
TOPOLOGY:linear  
15 MOLECULE TYPE:Other nucleic acid(synthetic DNA)  
SEQUENCE DESCRIPTION:SEQ ID NO:6:  
TTGTATCAAT CTCACTAGTT CTTCT 25  
20  
SEQ ID NO:7  
SEQUENCE LENGTH: 435  
SEQUENCE TYPE:amino acid  
25 STRANDEDNESS:single  
TOPOLOGY:linear  
30 MOLECULE TYPE:peptide  
ORIGINAL SOURCE:  
ORGANISM:Pyrococcus furiosus  
35 SEQUENCE DESCRIPTION:SEQ ID NO:7:  
Ala Lys Tyr Leu Glu Leu Glu Gly Gly Val Ile Met Gln Ala  
1 5 10 15  
40 Phe Tyr Trp Asp Val Pro Gly Gly Gly Ile Trp Trp Asp His Ile  
20 25 30  
45 Arg Ser Lys Ile Pro Glu Trp Tyr Glu Ala Gly Ile Ser Ala Ile  
35 40 45  
50 Trp Leu Pro Pro Pro Ser Lys Gly Met Ser Gly Gly Tyr Ser Met  
50 55 60  
55 Gly Tyr Asp Pro Tyr Asp Tyr Phe Asp Leu Gly Glu Tyr Tyr Gln

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	65	70	75
	Lys Gly Thr Val Glu Thr Arg Phe Gly Ser Lys Glu Glu Leu Val		
5	80	85	90
	Arg Leu Ile Gln Thr Ala His Ala Tyr Gly Ile Lys Val Ile Ala		
	95	100	105
10	Asp Val Val Ile Asn His Arg Ala Gly Gly Asp Leu Glu Trp Asn		
	110	115	120
	Pro Phe Val Gly Asp Tyr Thr Trp Thr Asp Phe Ser Lys Val Ala		
15	125	130	135
	Ser Gly Lys Tyr Thr Ala Asn Tyr Leu Asp Phe His Pro Asn Glu		
	140	145	150
20	Leu His Cys Cys Asp Glu Gly Thr Phe Gly Gly Phe Pro Asp Ile		
	155	160	165
25	Cys His His Lys Glu Trp Asp Gln Tyr Trp Leu Trp Lys Ser Asn		
	170	175	180
	Glu Ser Tyr Ala Ala Tyr Leu Arg Ser Ile Gly Phe Asp Gly Trp		
30	185	190	195
	Arg Phe Asp Tyr Val Lys Gly Tyr Gly Ala Trp Val Val Arg Asp		
	200	205	210
35	Trp Leu Asn Trp Trp Gly Gly Trp Ala Val Gly Glu Tyr Trp Asp		
	215	220	225
	Thr Asn Val Asp Ala Leu Leu Ser Trp Ala Tyr Glu Ser Gly Ala		
40	230	235	240
	Lys Val Phe Asp Phe Pro Leu Tyr Tyr Lys Met Asp Glu Ala Phe		
	245	250	255
45	Asp Asn Asn Asn Ile Pro Ala Leu Val Tyr Ala Leu Gln Asn Gly		
	260	265	270
	Gln Thr Val Val Ser Arg Asp Pro Phe Lys Ala Val Thr Phe Val		
50	275	280	285

Ala Asn His Asp Thr Asp Ile Ile Trp Asn Lys Tyr Pro Ala Tyr  
 290 295 300  
 5 Ala Phe Ile Leu Thr Tyr Glu Gly Gln Pro Val Ile Phe Tyr Arg  
 305 310 315  
 10 Asp Phe Glu Glu Trp Leu Asn Lys Asp Lys Leu Ile Asn Leu Ile  
 320 325 330  
 Trp Ile His Asp His Leu Ala Gly Gly Ser Thr Thr Ile Val Tyr  
 335 340 345  
 15 Tyr Asp Asn Asp Glu Leu Ile Phe Val Arg Asn Gly Asp Ser Arg  
 350 355 360  
 20 Arg Pro Gly Leu Ile Thr Tyr Ile Asn Leu Ser Pro Asn Trp Val  
 365 370 375  
 25 Gly Arg Trp Val Tyr Val Pro Lys Phe Ala Gly Ala Cys Ile His  
 380 385 390  
 Glu Tyr Thr Gly Asn Leu Gly Gly Trp Val Asp Lys Arg Val Asp  
 395 400 405  
 30 Ser Ser Gly Trp Val Tyr Leu Glu Ala Pro Pro His Asp Pro Ala  
 410 415 420  
 35 Asn Gly Tyr Tyr Gly Tyr Ser Val Trp Ser Tyr Cys Gly Val Gly  
 425 430 435  
 40

### Claims

1. An isolated DNA selected from the group consisting of:
  - (a) a DNA encoding a hyperthermostable  $\alpha$ -amylase and having a nucleotide sequence selected from the group consisting of the sequences represented by SEQ ID No: 1, SEQ ID No: 2 and SEQ ID No: 3 of the sequence listing;
  - (b) a DNA capable of hybridizing to the DNA of (a) under stringent conditions and which encodes hyperthermostable  $\alpha$ -amylase.

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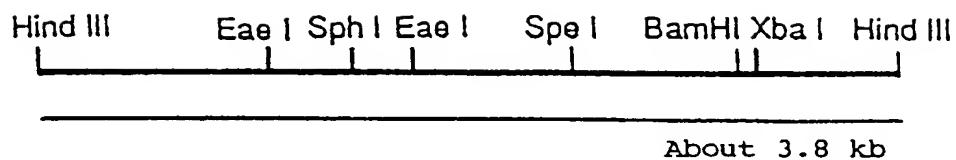


Fig. 1

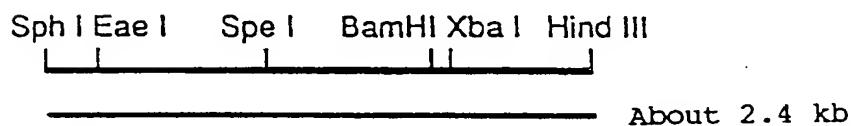


Fig. 2

26

AlaSerProValSerAlaAlaLysTyrLeuGluLeu  
 5'-GCAAGTCCAGTAAGTGCAGCAAAATACTTGGAGCTT-3'

AMFN 5'-CAGTAAGTG CAAAATACCTT-3'  
CCATGG  
 NcoI

Fig. 3

5'-GGATCAAAAGAAGACTAGTGAGATTGATACAAACTG-3'  
 SpeI  
 MRS 3'-TCTTCTTGATCACTCTAACTATGTT-5'

Fig. 4

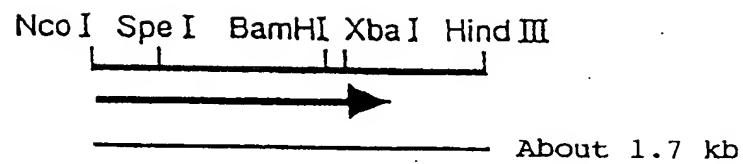


Fig. 5

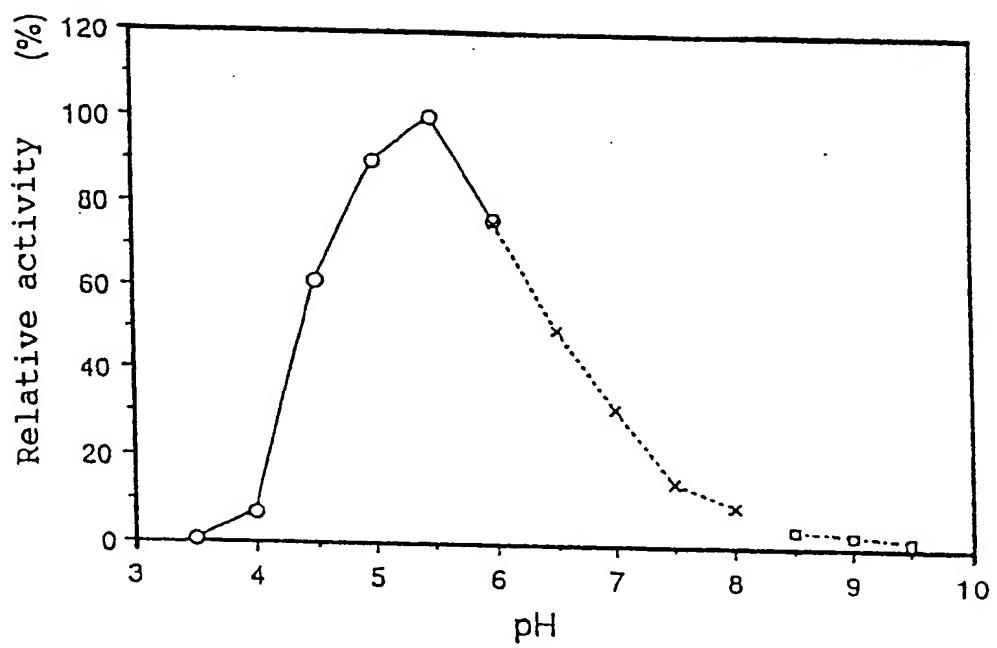


Fig. 6

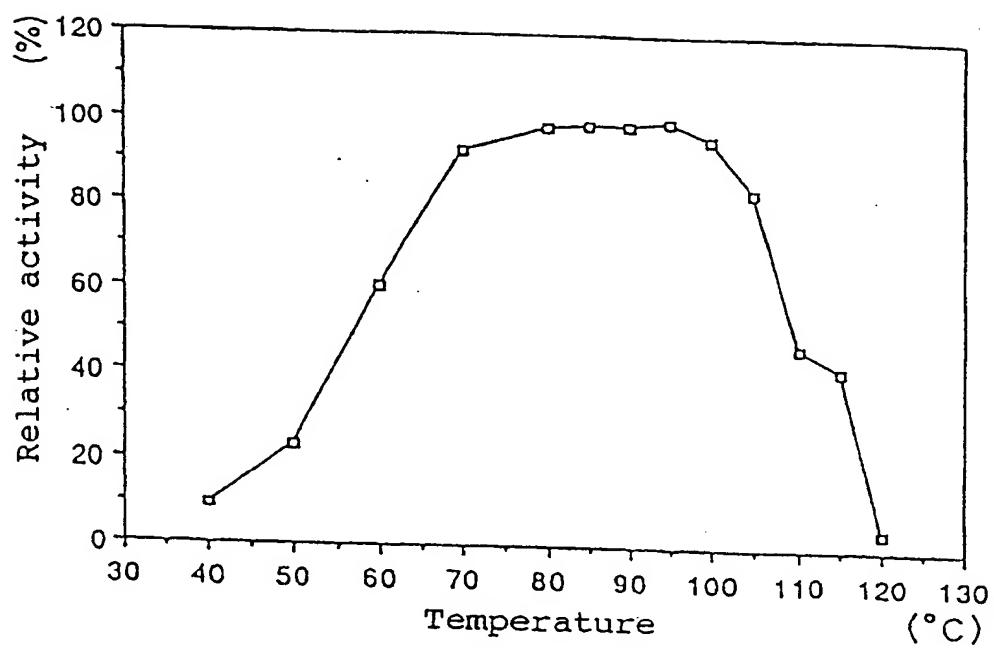


Fig. 7

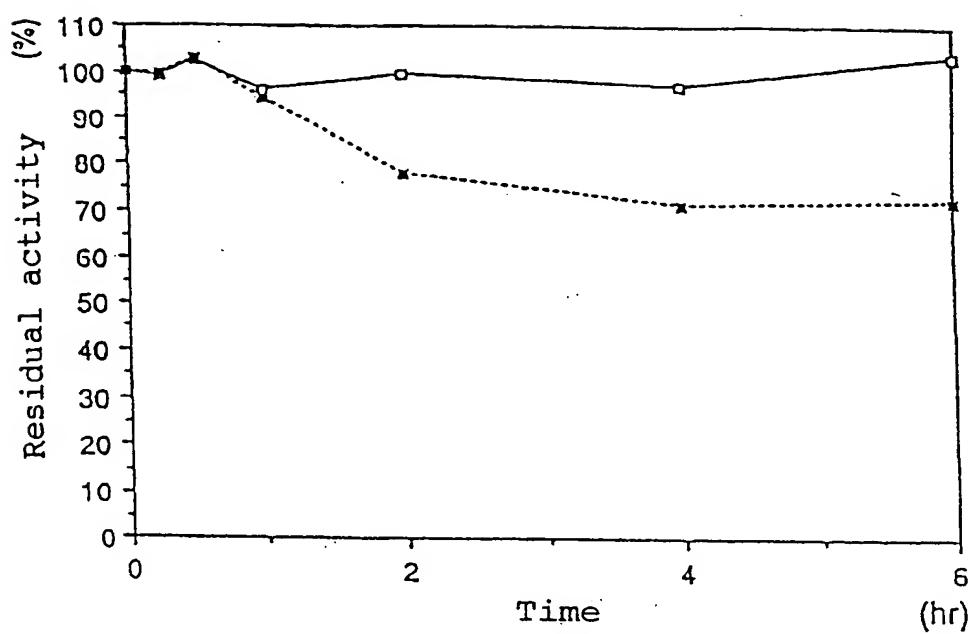


Fig. 8



European Patent  
Office

EUROPEAN SEARCH REPORT

Application Number  
EP 94 30 6856

DOCUMENTS CONSIDERED TO BE RELEVANT									
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)						
A,D	FEMS MICROBIOLOGY LETTERS, vol.71, 1990 pages 21 - 26 KOCH ET AL. 'Extremely thermostable amylolytic enzyme from the archaeabacterium Pyrococcus furiosus' * the whole document * ---	1	C12N15/56 //C12N9/28						
A,D	WO-A-90 11352 (NOVO-NORDISK A/S) 4 October 1990 * example 2 * ---	1							
A	EUROPEAN JOURNAL OF BIOCHEMISTRY, vol.174, no.1, May 1988 pages 15 - 21 FUKUSIMI ET AL. 'Cloning and sequencing of a heat-stable amylase gene from an anaerobic thermophile, Dictyoglomus thermophilum' * the whole document * ---	1							
T	EP-A-0 579 360 (THE JOHN HOPKINS UNIVERSITY; TAKARA SHUZO CO.LTD.) 19 January 1994 * the whole document * -----	1	TECHNICAL FIELDS SEARCHED (Int.Cl.6) C12N						
<p>The present search report has been drawn up for all claims</p> <table border="1"> <tr> <td>Place of search</td> <td>Date of completion of the search</td> <td>Examiner</td> </tr> <tr> <td>THE HAGUE</td> <td>9 January 1995</td> <td>Cupido, M</td> </tr> </table> <p><b>CATEGORY OF CITED DOCUMENTS</b></p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons &amp; : member of the same patent family, corresponding document</p>				Place of search	Date of completion of the search	Examiner	THE HAGUE	9 January 1995	Cupido, M
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